# THE EFFECTS OF SULFUR DIOXIDE UPON THE CHICK EMBRYO

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THE EFFECTS OF SULFUR DIOXIDE

UPON THE CHICK EMBRYO

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## SUMMARY

Sulfur dioxide was administered in chronic doses, 0.12, 1.08, 13.50 ppm and normal air, to fertile chicken eggs, from day zero of incubation to day 7.3. There was no significant effect on percent hatchability compared to the normal air group.

Sulfur-35 dioxide at a concentration of 0.01 ppm was employed as a tracer. The gas was administered from day zero of incubation to day 15. Sulfur-35 first entered the embryo between days three and six. It was found to be incorporated, primarily, into the cartilages and mucopolysaccharides.

Sulfur dioxide was administered in chronic doses of 1.08, 10.72, 13.50 ppm, and normal air, starting at day zero of incubation. Significant decreases in embryo weight were found at three of the four exposure periods. Decreases in embryo weight corresponded closely to increases in sulfur dioxide concentration.

Red blood cell pyruvate kinase activity was decreased by an acute dose (3000 ppm) of sulfur dioxide, administered from day 18 of incubation to day 19.5.

# CHAPTER I

## INTRODUCTION

In this century a new kind of catastrophe has been added to the roster: man-made air pollution trapped by atmospheric inversions.

The results of a study made in New York in 1963 show the existence of a serious hazard. It was determined that, during the previous year, 405 deaths and an unknown number of illnesses were attributable to air pollution which chronically hangs over the city. It is the present consensus that sulfuric acid and sulfur dioxide, a gas resulting from the combustion of sulfur, have been the principal factors in all air pollution disasters in recent history, most of which have been due to atmospheric inversions.

Sulfur dioxide was first purified and recognized as a definite compound by Joseph Priestly in 1775. It is colorless, twice as heavy as air, and has a strong suffocating odor, which is noticeable when sulfur is burned. Under standard conditions, one volume of water dissolves 79.8 volumes of the gas. It is easily condensed to a colorless liquid at -10°C and freezes to a snow-like solid at -72°C. In moist air or fogs, it combines with water to form a bisulfite anion (Alarie et al., 1973).

The cost of damages accruing from air pollution can not be precisely calculated, but it amounts to many billions of dollars a year. The direct costs of air pollution are estimated at $4.9 billion annually.

The painting of steel structures damaged by air pollution costs approximately $100 million a year. Damage to agricultural crops and livestock is computed to be $500 million a year. Adverse effects of air pollution on air travel cost from 40 to 80 million dollars a year (Hertzendorf, 1973).

The dollar value of medical costs and lost time from work due to air pollution is more difficult to assess. The Environmental Protection Agency (EPA) estimated that the cost of human mortality and morbidity from all air pollution is in the range of six billion dollars annually. The annual toll of air pollution on health, vegetation, materials, and property values has been estimated by the EPA at more than $16 billion each year, over 80 dollars for each person in the United States (Hertzendorf, 1973).

As the frequency of these crises increased, concerned groups began investigating the causes and effects of air pollution in order to define air quality standards. Wicken et al. (1964) determined an annual mean exposure to 0.44 parts per million (ppm) would produce adverse health effects. In an area of moderate particulate matter concentration, Brasser et al. (1967) found a range of 0.11 to 0.57 ppm, if sustained for three days, to be the lowest average daily concentration that could produce adverse effects.

The National Air Pollution Control Administration published its first air quality criteria in 1969. The current yearly mean level for sulfur dioxide is set at 0.03 ppm (Hertzendorf, 1973), which leaves a 30 percent margin of safety over the values determined by Wicken (1964). The 24 hour maximum mean concentration is 0.14 ppm, and is not to be ex-

ceeded more than once per year (Hertzendorf, 1973).

No literature was found concerning sulfur dioxide and chicken embryonic systems; however a considerable amount of research has been done on sulfur dioxide's effects upon the respiratory system of mammals. Although it is not within the scope of this study to examine these investigations, it is relevant to examine the existing research concerning other physiological events in animals exposed to sulfur dioxide and related compounds.

A substantial number of studies exist which use rats as the test animals. It has been shown that sulfite oxidase, the enzyme which catalyzes the oxidation of sulfite to sulfate, does not appear induceable by either bisulfite or sulfur dioxide in rat lungs (Cohen et al., 1973). Therefore, it is conceivable that high levels of bisulfite could saturate this system, producing bisulfite toxicity. Cohen et al. (1973) have calculated that the rate lung is capable of detoxifying at least 600 micromoles of bisulfite derived from inspired sulfur dioxide per day. This is equivalent to almost 20 ppm of sulfur dioxide in the atmosphere. Elfimova and Gusev (1969) exposed 15 rats to 0.19 ppm of sulfur dioxide for 96 days. They found significant reversible decreases in red blood cell count and nucleic acid concentration; slight dystrophic changes in the liver, heart, and kidneys; and reversible changes in large neurons of the pons, medulla oblongata, and cerebellum.

In rabbits, Bombart et al. (1973) found that an exposure of 35 ppm for one hour a day for seven months will significantly decrease the amount of free amino acids in the blood plasma. These results were comparable to rabbits living near an industrial complex for three years.

Enzyme studies conclude generally that multivalent anions are inhibitors of glycolysis (Webb, 1966). Dische and Ashwell (1955) have found that the anaerobic formation of lactate from glucose is depressed 84 percent by a 40 millimolar sulfate solution (in pigeon hemolyzates). It was concluded that there exist at least two sites of action, hexokinase and pyruvate kinase. The proposed modes of action are competitive inhibition with adenosine triphosphate or nicotinamide adenine dinucleotide and the complexing of magnesium by these anions, since several glycolytic enzymes are activated by this ion. Mettier et al. (1960) exposed four rabbits to 10 ppm for 32 days and found no effect on 6-phosphogluconate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, or lactate dehydrogenase.

In vivo studies on phage have demonstrated genetic mutations with a one molar bisulfite solution (Hayatsu and Muira, 1970a; Hayatsu et al., 1970b; Mukai et al., 1970). Shapiro et al. (1970) have demonstrated alterations in nucleic-acid bases in vitro with a one molar bisulfite solution. It has also been shown that this concentration of bisulfite can significantly decrease the ability of polyuridylic acid to form a double helical complex with polyadenylic acid and code for phenylalanine uptake into cell-free protein-synthesizing systems of Escherichia coli (Shapiro and Braverman, 1972).

The chicken embryo was chosen as the test animal for this study, primarily, because of the author's interest in the effects of sulfur dioxide upon embryonic systems and secondly, because of the isolated nature of this particular system. The purpose of this study was to examine the effects of sulfur dioxide upon the chick embryo. Specific

goals were (a) to determine if sulfur dioxide, at low and moderate concentrations, produces any effect on hatchability; (b) to discover if the gas reaches the embryo and if it is metabolized; (c) to determine if sulfur dioxide produces any change in embryo weight; (d) to determine if an acute dose has any gross effect upon the activity of pyruvate kinase in red blood cells of 19.5 days chick embryos.

# CHAPTER II

## MATERIALS AND METHODS

Four separate experiments were conducted to investigate the effects of sulfur dioxide upon chick development. The first experiment investigated sulfur dioxide's effect upon hatchability. The second determined if sulfur, from sulfur dioxide, enters the embryo and is metabolized. The third examined resultant change in embryo weight. The fourth experiment determined inhibition of pyruvate kinase in red blood cells.

Initially, four exposure chamber were constructed, employing 1.3 cm thick Plexiglas sheets and ethylene dichloride mixed with Plexiglas shavings as a cement. The inside dimensions of the chambers were 27.9 x 30.5 x 15.2 cm, with a volume of approximately 13 liters. Three of the four boxes were supplied with sulfur dioxide. The supply originated from permeation tubes, which were constructed of FEP Teflon containing sealed liquid sulfur dioxide (Analytical Instrument Development Inc., West Chester, Pennsylvania), which diffused through the porous tubes at a constant rate. The tubes were calibrated by measuring their weight loss over several hours at a constant temperature. This output remained constant until the liquid was nearly consumed. A constant concentration of sulfur dioxide was obtained by enclosing the tubes in glass pipe (Appendix A). Lab Crest Century flow meters were used to monitor gas-air flow rates (Fisher Scientific Company, Atanta, Georgia).

The four exposure chambers were arranged in a Percival (Boone,

Iowa) walk-in incubator maintained at 37°C. Several pans of water were placed in the incubator, stabilizing the humidity between 50 and 60 percent (wet-bulb dry-bulb method).

All eggs were Shaver-Starcross White-Leghorn obtained from KimberCHIK Hatcheries of Dixie (Atlanta, Georgia). Egg weights ranged from 45 to 78 grams after rounding to the nearest gram. Eggs weighing between 58 and 65 grams were selected in all experiments, in order to reduce variability. Except when noted, temperature was $37 \pm 0.5$°C.

## Hatchability

Two dozen eggs, within the designated weight range, were placed in each exposure chember on day zero of incubation. Flow rates were set at one liter per minute with sulfur dioxide concentrations of 13.50, 1.08, 0.12 and normal air averaging 0.007 ppm (personal communication, Mr. Weisenbaker, Fulton County Health Department).

The eggs were removed and candled after 7.4 days of continuous exposure. Infertile eggs were recorded and discarded. The fertile eggs were then transferred to a "Favorite Incubator" made by Leahy Manufacturing Company, Inc. (Higginsville, Missouri). Standard conditions of incubation were maintained, as recommended by the manufacturer (Appendix B), except that the eggs were turned every twelve hours during their turning period.

All chicks emerging from their shells without assistance prior to the end of twenty-two days of incubation were considered viable.

## Sulfur-35 Dioxide Tracing Experiment

One exposure chamber was modified in order to allow for monitoring of the temperature, relative humidity, and oxygen concentration (Oxygen Analyser Model 300, Scientific Products, Atlanta, Georgia). A magnetic stirring bar and motor were employed to circulate the air in the chamber. The magnetic stirring motor was placed outside the chamber and underneath the stirring bar. The exposure apparatus was contained in an incubator, manufactured by Acme Laboratory Equipment Company (New York, New York), which, in turn, was placed in a laboratory exhaust hood (Figure 1).

Five milliCuries (contained in five, one milliCurie vials) of sulfur-35 was obtained from New England Nuclear (Boston, Massachusetts). The specific activity of the gas was 22.4 milliCuries per millimole, with a radiometric purity of greater than 99 percent. On day zero of incubation, 17 eggs were sealed in the chamber and one vial of sulfur-35 dioxide was released, producing a concentration of 0.01 ppm (not including 0.007 ppm normally found in the air). The humidity, temperature,and oxygen concentration were monitored at least twice daily.

After three, six, nine, 12, and 15 days of exposure, the exposure atmosphere was drawn through a one molar sodium hydroxide solution, so as to remove any remaining sulfur-35 dioxide. The chamber was then opened and three eggs were removed. Another vial of sulfur-35 dioxide was placed in the chamber. The chamber was sealed, and the gas released.

One of the three eggs removed from the chamber was used for liquid scintillation counting of the embryo, shell, albumen, and yolk. The shell was ground to a fine powder using a mortor and pestal. The embryo was macerated, employing two "frosted" glass slides. A sample of the

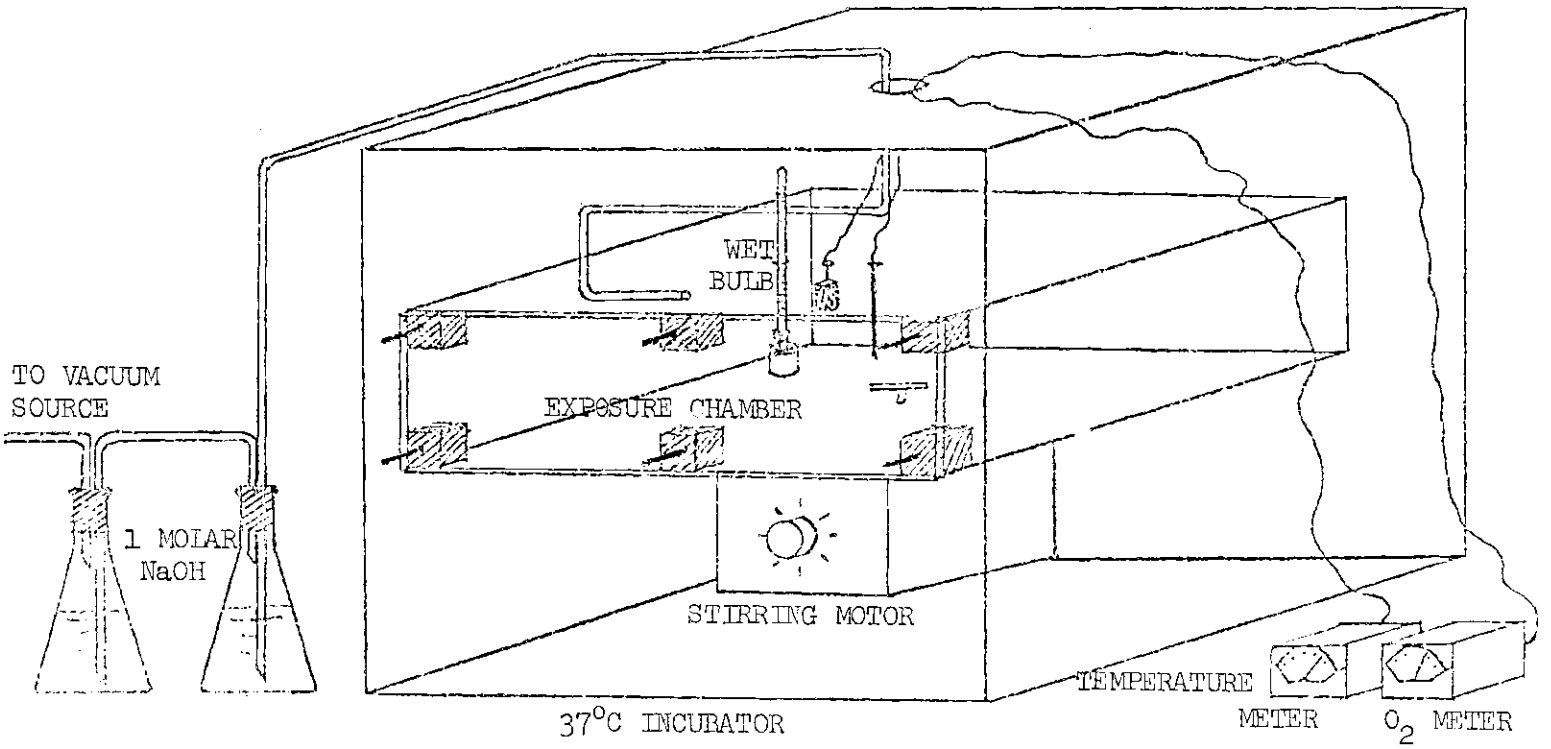



Figure 1. Exposure Apparatus For Sulfur-35 Dioxide Tracing Experiment.

albumen, yolk, and the entire embryo were dissolved, and a portion of the shell suspended, in 10.0 ml of the scintillation cocktail, Aquasol (New England Nuclear, Boston, Massachusetts). Three one-minute repititions were made on a Mark I Liquid Scintillation Counter, Model Number 6894 (Nuclear Chicago). Quenching was not determined for any of the samples, therefore, presence of sulfur-35 was reported on a yes or no basis. A second embryo was fixed for one-half hour in Bouin solution. The third egg was used only if one of the previous two was infertile or dead.

The second embryo, after fixation, was dehydrated and embedded in paraffin (Paraplast), according to the schedule in Appendix C. Serial saggital sections of five microns were prepared, using the Model 820 A.O. Spencer microtome. The slides were deparaffinated (Appendix D) and allowed to dry. They were then transferred to a photographic darkroom and coated with Eastman Kodak's $NTB_2$ photographic emulsion (Rochester, New York) according to Gude (1968). The slides were placed in wooden slide boxes containing calcium chloride, a drying agent. Photographic tape was applied to the boxes to prevent any light exposure. The boxes were then placed in a $10^{o}C$ refrigerator for a ten day exposure period. After exposure, the slides were developed (Appendix E) and stained with Delafield hematoxylin, counterstained with eosin (Appendix F), and mounted with Permamount.

This procedure was repeated at six, nine, 12, and 15 days. The 12 and 15 day embryos were large enough for dissection, Therefore, the heart, brain (occipital lobe), lung, kidney, stomach, and liver were removed and prepared for liquid scintillation and autoradiography. The

liquid scintillation and autoradiographic results were examined for time of entry into the embryo and distribution of the sulfur-35. Photomicrographs were taken of the autoradiographs exhibiting incorporation of the sulfur-35.

## Change in Embryo Weight

A series of four similar experiments was conducted to determine if sulfur dioxide has any effect on embryo weight, after periods of incubation accompanied by exposure to the gas.

The exposure periods for these experiments were 7.3, 8.9, 13.4, and 14.5 days. Twelve eggs were selected by weight and placed in each exposure chamber, which were contained in the Percival 37°C incubator. The humidity ranged from 50 to 60 percent. The sulfur dioxide was supplied by the permeation tubes in concentrations of 13.50, 10.72, 1.08, and normal air, averaging 0.007 ppm.

After the exposure period, the eggs were removed from the chambers, the embryos were extracted, and the allantois and extraembryonic membranes were removed. The embryos were weighed to the nearest thousandth of a gram on a Mettler H64 balance.

## Effect Upon Pyruvate Kinase

A single experiment was conducted to determine if sulfur dioxide has any inhibitory effect upon red blood cell pyruvate kinase. Four dozen eggs were selected by weight and incubated as described in Appendix B. On day 18 of incubation, 24 eggs were placed in a control chamber and 24 were placed in an exposure chamber supplied with approximately 3,000 ppm sulfur dioxide from a cylinder containing, pure, anhydrous sulfur

dioxide. Both chambers were contained in the Percival incubator and maintained at 37°C and 50 to 60 percent humidity. After 15 days the eggs from both chambers were removed and samples of blood were obtained. Blood from two embryos was combined and constituted a single sample.

A qualitative screening procedure for the detection of pyruvate kinase deficiency was obtained from Sigma Chemical Company (Saint Louis, Missouri). The blood samples were prepared according to the procedure recommended by Sigma (Appendix C). Due to the small quantity of reagent supplied in each vial, only eight tests could be run, four from the control and four from the experimental samples. Five microliter quantities were spotted on filter paper every five minutes for 90 minutes. After drying, the spots were examined under long wave ultraviolet light. These results were photographed with high speed Ektachrome daylight film (Eastman Kodak) through a Kodak E2 filter. The time at which each of the samples completed the reaction (lost fluorescence) was noted.

## Statistical Analysis

The statistical procedures employed are generally accepted methods as described by Steel and Torrie (1960). A slight modification in the additive partitioning of the hatchability data analysed by chi-square has been employed. The exact partitioning may be used to test orthogonal contrasts of a particular type, each with one degree of freedom. The resulting component chi-square values computed will add exactly to the total chi-square as computed in the usual way (Kastembaum, 1960). The data obtained from the hatchability study fit a 2 x 4 contingency table. If two adjacent exposure levels were compared and a significant chi-square value was obtained, then the next comparison was made. If,

however, two adjacent exposure levels were not different, then those two cells were collapsed and the resulting pool of these two groups was compared with the next exposure level.

# CHAPTER III

# RESULTS AND DISCUSSION

## Hatchability

The results of the hatchability study (Tables 1 and 2) indicated that sulfur dioxide, at the concentrations and durations of exposure used, produced no significant effect upon egg hatchability. However, there does exist a trend indicating a decrease in viability with increased levels of exposure. Because of these results, it was proposed that the sulfur dioxide did not reach the embryo during the 7.4 day exposure period, or that sulfur dioxide did reach the embryo and produced no effect under the conditions of this experiment. It was determined that a sulfur-35 dioxide tracing experiment would help to answer this question.

## Sulfur-35 Dioxide Tracing Experiment

The viability of the embryos in the sulfur-35 dioxide tracing experiment (at 0.01 ppm) was too low to obtain results for all five three-day samples. This low viability may have been the result of the variation in incubation temperature (Appendix H).

Results of the liquid scintillation are presented in Appendix I. After three days of exposure, it was evident that sulfur-35 was present in the yolk, albumen, and shell. The three day embryo contained nonsignificant levels compared to background (t-test on the three, one minute counts), suggesting an absence of sulfur-35 in the embryo at this time

After six days of exposure the yolk, albumen, and shell contained

Table 1. Effect of Three Concentrations of Sulfur Dioxide on Egg Hatchability After 7.4 Days of Exposure

| $SO_2$ (ppm) | Number of Fertile Eggs | Number of Eggs Hatched | Percent Hatch |
|---|---|---|---|
| Control | 21 | 20 | 95.2 |
| 0.12 | 22 | 20 | 90.9 |
| 1.08 | 21 | 19 | 90.5 |
| 13.50 | 21 | 18 | 85.7 |

Table 2. Chi-square Comparisons on the Effects of Three Concentrations of Sulfur Dioxide on Egg Hatchability After 7.4 Days of Exposure

| Comparison | Chi-square |
|---|---|
| C vs 0.12 ppm | 0.24 |
| C, 0.12 ppm vs 1.08 ppm | <0.01 |
| C, 0.12 ppm, 1.08 ppm vs 13.50 ppm | <0.01 |

Notes:

1. All Chi-square values are not significant at the 0.05 level, one degree of freedom.

significant quantities of tracer (Appendix I). Representative photomicrographs of the autoradiographs (ARGs) exhibiting incorporation of sulfur-35 are presented in Figures 2a,b,c, and d. The six day ARGs demonstrated the presence of sulfur-35 in the following structures, in order of decreasing amounts; mesodermal core of the limb bud, sclera-tome (Figure 2a), yolk and epithelium of the stomach (Figure 2b), myocardium (Figure 2c), mesonephric kidney, mesenchyme of the limb bud, wall of the brain and the liver.

Autoradiographs prepared from the nine day embryo demonstrated the incorporation of sulfur-35 in the following structures, in order of decreasing amounts; vertebrae, intestinal epithelium, brain wall, and bronchioles.

Autoradiographs of the 12 day embryo exhibited evidence of isotope incorporation only in the sternum and liver. It is the author's opinion that the low quantity of exposed grains in the 12 day embryo were the consequence of an error during ARG development.

The 15 day embryos contained significant levels of sulfur-35 in all tissues examined with liquid scintillation (Appendix I). Results of the 15 day ARGs showed high levels in the epithelium of the stomach (Figure 2d) and cartilages, less in the myocardium of the heart, and small quantities in the liver.

As a summary of the sulfur-35 tracing experiment it is the author's opinion that much of the sulfur dioxide is trapped in the shell, yolk, and albumen during the initial 24 to 72 hours of exposure. It should be noted that the chorio-allantois is not functional until the fourth day of incubation (Patten, 1971). From this experiment, it is apparent

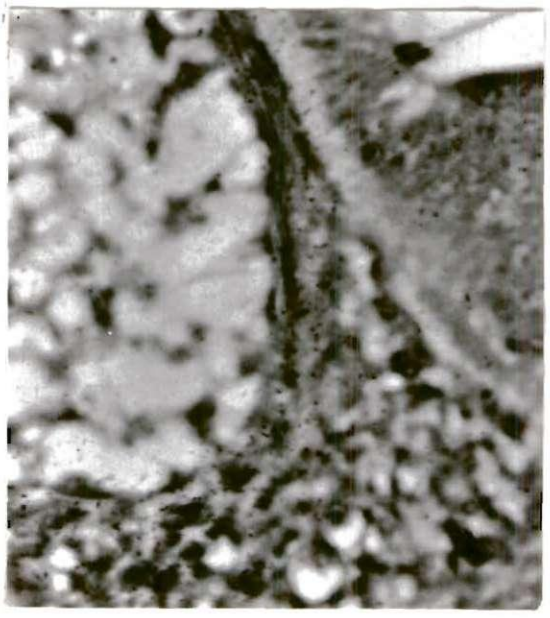


Figure 2a. Six Day Tail, S-35 in Scleratome, x-section, x400.

Figure 2b. Six Day Stomach, S-35 in Yolk and Epithelium, x-section, x400.

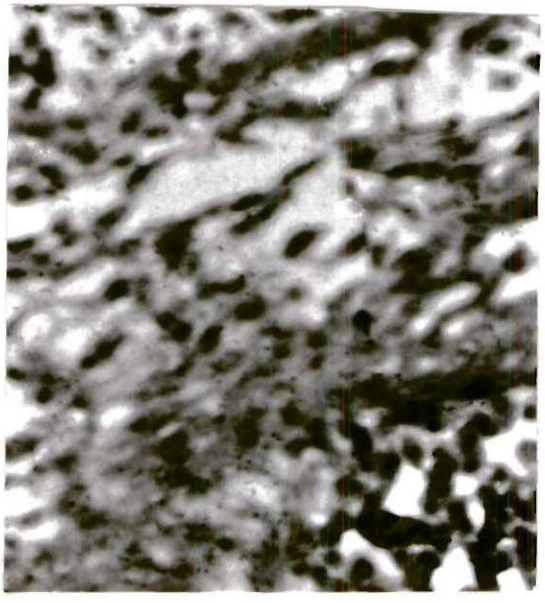


Figure 2c. Six Day Heart, S-35 in Myocardium, x400.

Figure 2d. Fifteen Day Stomach, S-35 in Yolk and Epithelium, x-section, x400.

that the sulfur-35 reaches the embryo between the third and sixth days of incubation. It is plausible to suggest that with the onset of a functional chorio-allantois, the sulfur dioxide passes through the shell and enters the embryo at a much faster rate than could be possible prior to this time. As the yolk is digested, and the albumen consumed, more of the sulfur can enter the embryo. This is evidenced by the high levels found in the epithelial lining of the stomach and yolk adjacent to it (Figure 2b). Additional evidence for the sulfur-35 entering the embryo between the third and sixth days of incubation is the absence of significant numbers of exposed grains in the notochordal tissue in the tail of the six day embryo (Figure 2a). It is known that the notochord is fully developed by the end of the second day of incubation (Patten, 1971). If the sulfur-35 was present, in the form of sulfate, prior to this time, it would be incorporated into the notochord in one of more of the forms of chondroitin sulfate. However, this does not occur and it can be seen that the sulfur-35 is incorporated into the scleratome of the somites. This scleratome rapidly develops into the axial skeleton subsequent to the sixth day of incubation (Patten, 1971).

## Change in Embryo Weight

The results of the embryo weight experiment are presented in Table 3. The omissions in Table 3 at 8.9 and 14.5 days were the result of a malfunction of a flow meter in one case, and the permeation tube falling out of its holder in the other.

It can be seen from Table 3 that after 7.3 days of exposure the experimental embryos all weighed significantly less than the controls.

Table 3. A Summary of Sulfur Dioxide's Effect Upon Embryo Weight (g) at Different Durations of Exposure

| | $SO_2$ (ppm) | Days of Exposure | | | |
|---|---|---|---|---|---|
| | | 7.3 | 8.9 | 13.4 | 14.5 |
| Mean<br>S.D.<br>N | Control | $\frac{0.217^{a}}{0.024}$<br>10 | $\frac{0.525^{a}}{0.062}$<br>6 | $\frac{2.406^{a}}{0.176}$<br>7 | $\frac{4.708^{a}}{0.350}$<br>10 |
| Mean<br>S.D.<br>N | 1.08 | $\frac{0.188^{b}}{0.019}$<br>8 | -- | $\frac{2.230^{ab}}{0.192}$<br>8 | $\frac{3.933^{b}}{0.417}$<br>9 |
| Mean<br>S.D.<br>N | 10.72 | $\frac{0.167^{b}}{0.010}$<br>5 | $\frac{0.481^{a}}{0.050}$<br>7 | $\frac{2.139^{b}}{0.173}$<br>10 | -- |
| Mean<br>S.D.<br>N | 13.50 | $\frac{0.180^{b}}{0.025}$<br>8 | $\frac{0.494^{a}}{0.052}$<br>3 | $\frac{2.215^{ab}}{0.198}$<br>4 | $\frac{3.435^{c}}{0.313}$<br>6 |

Notes:

1. Means having the same superscript are not significantly different at the 0.01 level using Duncan's Multiple Range Test.
2. S.D. is the Standard Deviation.
3. N is the Number of viable embryos.

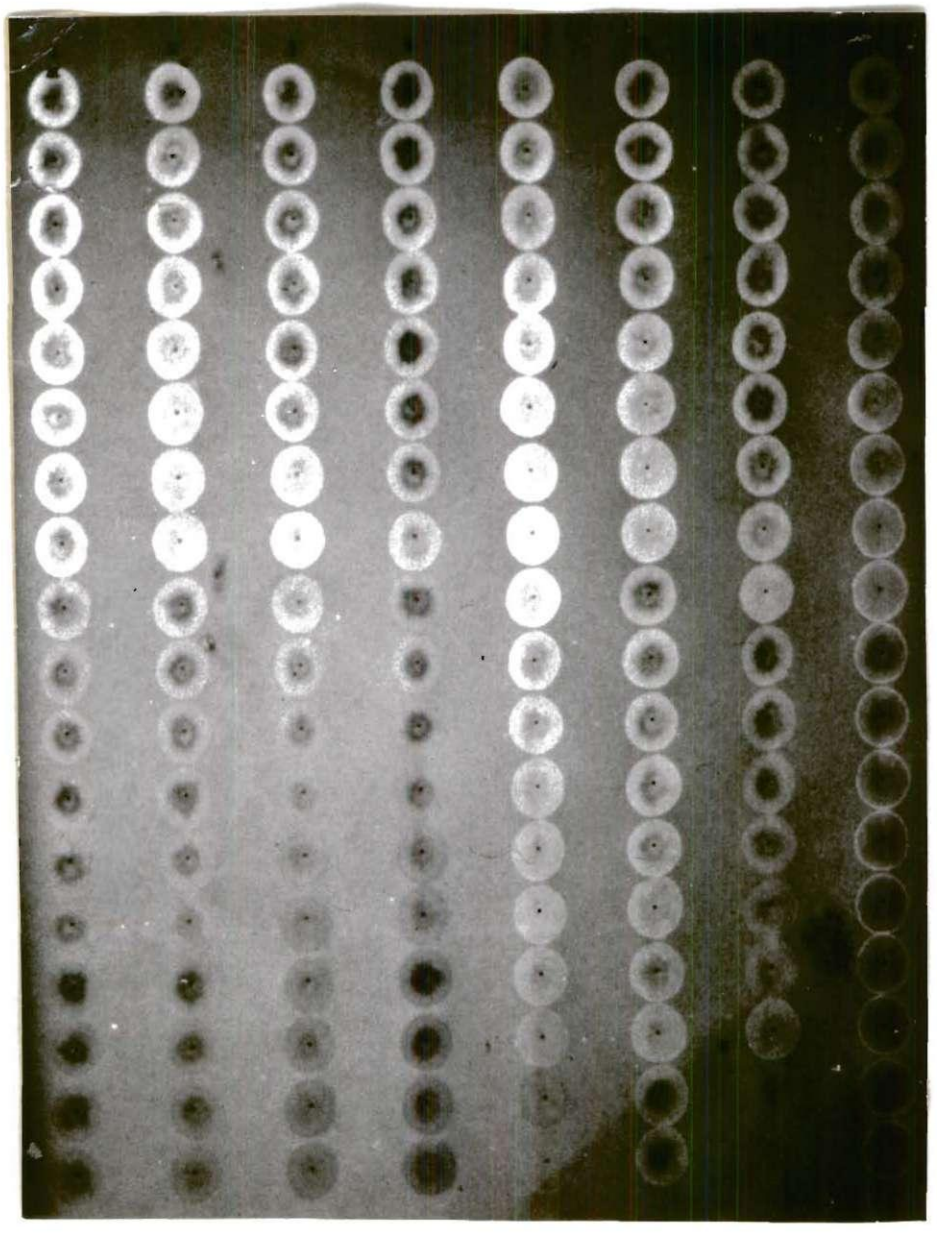
Not being significantly different from each other, they comprise a single group. At 8.9 days none of the groups were significantly different, suggesting some compensating mechanism within the embryo. After 13.4 days the means separated into two groups, with embryos maintained at 10.72 ppm being significantly different from all other means. At 14.5 days the three groups obtained were all significantly different from each other. It can be inferred that there is more than one mechanism mediating sulfur dioxide effects on the weights of the embryos; since the weights of the experimental groups at 7.3 days are significantly different from the control group, and this difference is abolished by 8.9 days and gradually reappears by 14.5 days.

In conclusion, it is apparent that sulfur dioxide has the potential to decrease embryo weight even at low concentrations. It may also be hypothesized that there is more than a simple mechanism mediating sulfur dioxide effects.

## Pyruvate Kinase

The results of the pyruvate kinase experiment (Figure 3) appear to be conclusive. After 75 minutes, all of the fluoresence had depleted from the dried spots of the reaction mixture in the four control samples, indicating that the pyruvate kinase was able to catalyse the conversion of phosphoenolpyruvate to pyruvate. While at the same point in time, all four of the experimental samples retained a visible amount of fluoresence.

If sulfur dioxide had no effect upon pyruvate kinase, then at the time when four of the eight samples had completed the reaction (lost



CONTROLS EXPERIMENTALS

Figure 3. Effects of Sulfur Dioxide upon Pyruvate Kinase. Five minutes separate each row.

fluoresence). it would be expected that two of the completed reactions would be from the controls and two from the experimentals. It can be seen that sulfur dioxide causes the pyruvate kinase to be inhibited in some manner. It is important to note that glycolysis, in red blood cells, is the major pathway for the cell to obtain energy, and any decrease in glycolysis could have significant effects on the cells ability to perform its normal functions in the blood.

Dische and Ashwell (1955) indicated that the anaerobic formation of lactate from glucose is depressed 84 percent in pigeon hemolyzates by a 40 millimolar sulfate solution. It was suggested that hexokinase and pyruvate kinase were inhibited. One of the explanations proposed the formation of a sulfate chelate with the divalent magnesium cation, a cofactor for the kinases.

# CHAPTER IV

## CONCLUSIONS

Within the limitations of these experiments it can be concluded that:

a) Sulfur dioxide has no significant effect upon the hatchability of chicken embryos when administered, starting at day zero of incubation and continuing for 7.4 days, at concentrations as high as 13.50 ppm.

b) From autoradiographic and liquid scintillation results, it was determined that sulfur-35 initially in the form of sulfur-35 dioxide, at a concentration of 0.01 ppm, administered from day zero of incubation through day fifteen, first enters the embryo between days three and six. Of the tissues examined with autoradiography, the sulfur-35 was, generally, incorporated into the cartilages, myocardium, brain wall, and epithelium of the stomach.

c) Sulfur dioxide, when administered from day zero of incubation will cause a decrease in embryo weight at concentrations of 1.08, 10.72, and 13.50 ppm, after 7.3 days of incubation.

d) When sulfur dioxide is administered in acute doses, 3000 ppm, starting at day 18 of incubation and continuing until day 19.5 will cause a decrease in red blood cell pyruvate kinase activity.

# APPENDIX A

## CALCULATION OF PPM FOR A GAS

$$C = (R/F) \times (22.4/M) \times (T/273) \times (760/P)$$

Where:

C = exit gas concentration (ppm)

R = permeation rate (ng/min)

F = air flow rate (ml/min)

M = molecular weight of the gas (g)

T = temperature of F ($K^{o}$)

P = pressure at which F is measured (mm Hg), the yearly mean pressure for Atlanta, Georgia was used in the calculation (735.84 mm Hg)

# APPENDIX B

## STANDARD CONDITIONS OF CHICKEN EGG INCUBATION[a]

1. Incubation period: 21 days
2. Operating temperature: 100°F
3. Wet-bulb reading during turning period: 85-87°F
4. Wet-bulb reading after completion of turning period: 90-94°F
5. Length of turning period: 18 days
6. Eggs turned three times daily during turning period[b]
7. Incubator ventilation required on 10th day
8. Egg positioners used until completion of turning period

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[a] Recommended by Leahy Manufacturing Company, Inc., Higginsville, Missouri.

[b] Eggs were turned twice daily during turning period.

# APPENDIX C

## TISSUE PREPARATION: SCHEDULE FOR FIXATION, DEHYDRATION, AND INFILTRATION[a]

1. Fix one-half hour in Bouins
2. Transfer to 50% ethyl alcohol: 1 hour
3. Transfer to 70% ethyl alcohol: 1 hour
4. Transfer to 95% ethyl alcohol: 1 to 1½ hours
5. Transfer to absolute ethyl alcohol (2 changes): ½ to 1 hour
6. Transfer to absolute ethyl alcohol/xylene (1:1): ½ to 1 hour
7. Transfer to xylene (2 changes): ½ to 1 hour
8. Transfer to xylene/paraffin (1:1): ½ to 1 hour
9. Transfer to paraffin (2 changes): ½ to 1 hour
10. Embed

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[a]Humason, 1972

# APPENDIX D

## STEPS FOR REMOVAL OF PARAFFIN[a]

1. Xylene (2 changes): 5 minutes
2. Absolute ethyl alcohol (2 changes): 3 minutes
3. 95% ethyl alcohol: 3 minutes
4. 70% ethyl alcohol: 3 minutes
5. Distilled water: 3-5 minutes

---

[a]Gude, 1968

# APPENDIX E

## EMULSION DEVELOPING PROCEDURE[a]

1. Developer D-19 (Eastman Kodak Company): 6 minutes
2. Distilled water: dip 8-10 times
3. Kodak Fixer (Eastman Kodak Company): 10-15 minutes

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[a] Gude, 1968

# APPENDIX F

## STAINING AND CLEARING PROCEDURE[a]

1. Delafield's hematoxylin: 4 minutes
2. Water: dip 8-10 times
3. Lithium carbonate, saturated aqueous solution: 1-2 minutes
4. Water: dip 8-10 times
5. 70% ethyl alcohol: 2-3 minutes
6. Eosin, 1% in 90% ethyl alcohol: 1-2 minutes
7. 95% ethyl alcohol (2 changes): 2-3 minutes
8. Absolute ethyl alcohol: 2-3 minutes
9. Ethyl alcohol and xylene (1:1): 5-10 minutes
10. Xylene: 5-10 minutes
11. Permamount

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[a]Modified from Gude, 1968

# APPENDIX G

## A QUALITATIVE SCREENING PROCEDURE FOR THE DETECTION OF PYRUVATE KINASE DEFICIENCY IN RED BLOOD CELLS[a]

1. Centrifuge whole blood samples for 10-15 minutes at approximately 3000 rpm
2. Carefully aspirate off the plasma and buffy coat
3. Prepare a 20% suspension of red cells in saline by adding 4 volumes of 0.9% sodium chloride solution to 1 volume of packed cells and mix
4. Similarly prepare a 20% suspension from a sample of "normal" blood
5. Reconstitute a Pyruvate Kinase Deficiency Screening Test Reagent vial, with 2.0 ml of water
6. Label Control and Experimental Test tubes
7. Into each tube pipet: 0.2 ml of red cell suspension prepared from Control blood samples, mix by swirling
8. a) To Control add: 0.02 ml of red cell suspension from control blood
   b) To Experimental add: 0.02 ml of red cell suspension from experimental blood
9. Immediately spot drops from the mixtures onto a piece of filter paper (5 $\mu$l quantities were applied with a microliter syringe), place in 37$^{o}$C water bath
10. Again spot drops onto filter paper at 5 minute intervals
11. Allow the filter paper to thoroughly air dry (about 10-15 minutes)
12. After spots have dried, inspect under long-wave UV light

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[a]Sigma Technical Bulletin No. 205

# APPENDIX H

## INCUBATION TEMPERATURE DURING THE S-$35O_2$ TRACING EXPERIMENT

| Day | Temperature ($^{o}$C) | Day | Temperature ($^{o}$C) |
|---|---|---|---|
| 1 | 39.8 | 9 | 37.2 |
| 2 | 38.2 | 10 | 37.5 |
| 3 | 36.8 | 11 | 37.2 |
| 4 | 36.2 | 12 | 36.5 |
| 5 | 37.0 | 13 | 36.8 |
| 6 | 36.1 | 14 | 37.6 |
| 7 | 33.4 | 15 | 37.0 |
| 8 | 36.5 | | |

# APPENDIX I

## A SUMMARY OF SULFUR-35 DIOXIDE LIQUID SCINTILLATION

| Sample | Exposure Period (days) | Sulfur-35 |
|---|---|---|
| embryo | 3 | no |
| albumen | 3 | yes |
| yolk | 3 | yes |
| shell | 3 | yes |
| embryo | 6 | --[a] |
| albumen | 6 | yes |
| yolk | 6 | yes |
| shell | 6 | yes |
| heart | 15 | yes |
| brain | 15 | yes |
| lung | 15 | yes |
| kidney | 15 | yes |
| stomach | 15 | yes |
| liver | 15 | yes |

[a]Embryo died before six days.

Note:

1. Positive responses in sulfur-35 column are significantly above background level.

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